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Putrescine reduces antibiotic-induced oxidative stress as a mechanism of modulation of antibiotic resistance in *Burkholderia cenocepacia*

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Communication of antibiotic resistance among bacteria via small molecules is implicated in transient reduction of bacterial susceptibility to antibiotics, which could lead to therapeutic failures aggravating the problem of antibiotic resistance. Released putrescine from the extremely antibiotic resistant bacterium *Burkholderia cenocepacia* protects less resistant cells from different species against the antimicrobial peptide polymyxin B (PmB). Exposure of *B. cenocepacia* to sub-lethal concentrations of PmB and other bactericidal antibiotics induce reactive oxygen species (ROS) production and expression of the oxidative stress response regulator OxyR. We evaluated whether putrescine alleviates antibiotic-induced oxidative stress. The accumulation of intracellular ROS such as superoxide ion and hydrogen peroxide was assessed fluorometrically with dichlorofluorescein diacetate, while the expression of OxyR and putrescine synthesis enzymes was determined in luciferase assays using chromosomal promoter-*lux* reporter system fusions. We evaluated wild type and isogenic deletion mutant strains with defects in putrescine biosynthesis after exposure to sub-lethal concentrations of PmB and other bactericidal antibiotics. Exogenous putrescine protected against oxidative stress induced by PmB and other antibiotics, whereas reduced putrescine synthesis resulted in increased ROS generation, and a parallel increased sensitivity to PmB. Of the 3 *B. cenocepacia* putrescine synthesizing enzymes, PmB induced only BCAL2641, an ornithine decarboxylase. This study exposes BCAL2641 as a critical component of the putrescine-mediated communication of antibiotic resistance, and as a plausible target for designing inhibitors that would block the communication of such resistance among different bacteria, ultimately reducing the window of therapeutic failure in treating bacterial infections.

INTRODUCTION

The relentless increase in multidrug resistance, particularly intrinsic, high-level resistance, undermines new treatments improving health and extending the life of patients especially of those with chronic conditions (1). For example, respiratory failure secondary to chronic pulmonary bacterial infection in patients with cystic fibrosis hinders the dramatic improvements in survival achieved over the last several decades and remains the primary cause of death (2). The emergence of growing numbers of cystic fibrosis pathogens with intrinsic, multidrug resistance such as *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, and nontuberculous mycobacteria creates a further need for novel therapies (2). We investigate the mechanisms of high-level intrinsic multidrug resistance using *Burkholderia cenocepacia* as a model bacterium. *B. cenocepacia* is an environmental, opportunistic pathogen that belongs to the *B. cepacia* complex and causes serious respiratory infections in CF patients (3). These infections are associated with faster decline in lung function, debilitating exacerbations and ultimately death (4-6), and they also reduce the survival of CF patients after lung transplant (7).

While genetic mechanisms are considered the quintessential means of transfer of antibiotic resistance traits among bacteria, small molecules are also capable of modulating the antibiotic response of bacteria (8). The clinical outcome of antibiotic treatment does not always correlate with the expectations based on *in vitro* susceptibility testing performed on individual clinical isolates (9). Owing to the polymicrobial nature of many infections (10), cross-talk between the different bacterial species is likely to occur during infection. Such chemical communication of antibiotic resistance among bacteria may aggravate the problem of antibiotic resistance by potentially causing transient reduction in the susceptibility to antibiotics, potentially leading to

therapeutic failures. For example, a transient increase in resistance to antimicrobial peptides by exposure to host polyamines was shown for the urogenital pathogen *Neisseria gonorrhoeae* (11). Identifying chemical communicators of antibiotic resistance and their mechanism of protection would provide another avenue for intervention to combat the increase and spread of antimicrobial resistance. Recently, we demonstrated that *B. cenocepacia* exhibits a non-genetic mechanism to reduce antibiotic susceptibility that is chemically mediated by putrescine and YceI, a small secreted protein of unknown function that is highly conserved in bacteria (12). Putrescine is a polyamine produced by almost all living organisms (13). When released from *B. cenocepacia*, putrescine protects less resistant cells from the same and different species from the antimicrobial peptide polymyxin B (PmB) (12).

The mechanism of protection is partly due to the ability of putrescine to compete with PmB for binding to the surface of *B. cenocepacia* (12). However, polyamines can also quench oxidative species (14) and protect membranes from lipid peroxidation (15). Various classes of antibiotics induce oxidative stress and increased production of reactive oxygen species (ROS) (16-19). Although the specific lethal role of ROS generated in response to antibiotics remains under discussion (16, 20, 21), oxidative stress constitutes a burden on the bacterial cells (22). Therefore, it is conceivable that protection from oxidative stress accompanying antibiotic exposure would improve the bacterial response to antibiotics, thus increasing resistance.

Here we show that when present at sub-lethal concentrations, PmB and other bactericidal antibiotics induce oxidative stress in *B. cenocepacia*. Our findings revealed that exogenous and endogenous putrescine protects against antibiotic-mediated oxidative stress. This work exposes another mechanism of putrescine-mediated protection from antibiotics alongside with protection of cell surface from binding of PmB previously described (12). By examining the expression

94 patterns of the different putrescine synthesizing enzymes in response to antibiotics, we
95 discovered that the ornithine decarboxylase BCAL2641 is a plausible target for designing
96 inhibitors that would block putrescine-mediated communication of antibiotic resistance among
97 different bacteria, ultimately reducing the window of therapeutic failure in treating bacterial
98 infections.
99

MATERIALS AND METHODS

Strains and reagents. Table 1 lists bacteria and plasmids used in this study. Bacteria grew in LB at 37°C. Antibiotics (Sigma, St Louis, MO, USA) were diluted in water except for PmB, which was diluted in 0.2% bovine serum albumin/0.01% glacial acetic acid buffer. Rifampicin was dissolved in dimethyl sulphoxide (DMSO).

General molecular techniques. DNA manipulations were performed as previously described (23). T4 DNA ligase (Roche Diagnostics, Laval, Quebec, Canada), Antarctic phosphatase (New England Biolabs, Pickering, Ontario, Canada) and restriction endonucleases (Roche or New England Biolabs) were used as recommended by the manufacturers. Transformation of *Escherichia coli* GT115 was performed using the calcium chloride method (24). Mobilization of plasmids into *B. cenocepacia* was conducted by triparental mating (25) using *E. coli* DH5 α carrying the helper plasmid pRK2013 (26). DNA amplification by polymerase chain reaction (PCR) was performed using a C1000 Thermal cycler (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada) with Taq or HotStar HiFidelity DNA polymerases (Qiagen, Mississauga, Ontario, Canada) and optimized for each primer pair. DNA sequencing was carried out at the DNA sequencing Facility of York University, Toronto, Canada or at Eurofins MWG Operon, Huntsville, Alabama, USA. The DNA sequences were analyzed with the BLAST computer program and compared to the sequenced genome of *B. cenocepacia* strain J2315.

Fluorometric determination of ROS. Overnight cultures of the parental *B. cenocepacia* K56-2 and the appropriate mutants in LB medium were diluted to an optical density at 600 nm (OD₆₀₀) of 0.1 in fresh medium. Five-ml aliquots were incubated at 37°C for 3 h at 200 rpm. Antibiotics and/or putrescine were added at the specified concentrations and the cultures were

further incubated at 37°C for 2 h at 200 rpm. After incubation, the OD₆₀₀ was measured and aliquots containing cells equivalent to an OD₆₀₀ of 0.4 were pelleted, washed with phosphate buffered saline (PBS), and resuspended in 1 ml of PBS. Superoxide radicals and other ROS were determined by diluting the obtained suspension 100 fold in 1 ml PBS and adding 2',7'-dichlorofluorescein diacetate (DCF) to a final concentration of 2 µM. The reaction mixture was then incubated at 37°C for 30 min with rotation. After incubation, the fluorescence was measured in 200-µl aliquots placed into 96-well white plates (Microfluor-2 White, Thermo Scientific) at λ_{ex}= 480 nm and λ_{em}= 521 nm, using Cary Eclipse fluorescence spectrophotometer (Varian, Inc., Mississauga, Ontario, Canada). In addition, the OD₆₀₀ of the same suspensions were measured and used to normalize the fluorescence values. Hydroxyl radical production was determined in 600 µl bacterial suspensions without dilution using 3'-(*p*-hydroxyphenyl) fluorescein (HPF) at a final concentration of 5 µM. Fluorescence was measured at λ_{ex}= 495 nm and λ_{em}= 530 nm in 200 µl aliquots placed into 96-well white plates. Background fluorescence of each probe in buffer control was subtracted. Autofluorescence of the bacterial suspensions, without adding the probes, was measured and corrected for by subtraction from the fluorescence signals. Data were normalized to the OD₆₀₀ of the bacterial suspensions. The suspensions were protected from light throughout the assays to avoid photo-oxidation.

Antibiotic susceptibility testing. Overnight cultures of the parental *B. cenocepacia* K56-2 and the appropriate mutants in LB medium were diluted to an optical density at 600 nm (OD₆₀₀) of 0.0008 (low inoculum) or 0.04 (high inoculum) in fresh LB medium and 0.04 in fresh M9 minimal medium with or without the antibiotic and incubated at 37°C with medium continuous shaking in a Bioscreen C automated growth curve analyzer (MTX Lab Systems, Vienna, VA, USA). Bacterial growth was assessed turbidimetrically at 600 nm.

***In vitro* antioxidant activity assay.** The ability of putrescine to scavenge free radicals was determined using a system of *in vitro* generation of superoxide radicals containing phenazine methosulfate (PMS)-NADH as previously described (27). Briefly, the reaction mixture consisted of 21 mM phosphate buffer (pH 8.3), 0.7 mM NADH, 17 μ M nitro blue tetrazolium, and the corresponding quantity of putrescine. The reaction was initiated by adding 4 μ M PMS. The reaction mixtures were mixed and the amount of formazan formation was measured immediately using the spectrophotometer at 560 nm. The percentage of inhibition of formazan formation by putrescine was calculated relative to the control lacking putrescine.

Transcriptional fusions to *luxCDABE*. The promoter regions from BCAL2641, BCAM1111, BCAM1112 and OxyR were PCR amplified. The PCR products were digested with *Eco*RI and cloned into the *Eco*RI digested and dephosphorylated pGSVTp-*lux* plasmid. The orientation of the promoter region was checked by PCR and luminescence of *E. coli* GT115 colonies carrying the plasmids. The resulting plasmids contained the promoter region of the genes of interest fused to the *luxCDABE* reporter system. The plasmids were mobilized into K56-2 and the appropriate mutants by triparental mating. Transconjugants (carrying the chromosomal promoter-reporter fusions) were selected on LB agar plates containing 100 μ g/ml of trimethoprim (Tp), 200 μ g/ml ampicillin and 10 μ g/ml gentamicin.

Luminescence expression assays. Overnight cultures in LB containing 100 μ g/ml Tp were diluted into fresh LB medium to OD₆₀₀=0.04. After addition of the antibiotics and/or putrescine, 300 μ l of sample were loaded in triplicate, for each time-point, in a 100-well honeycomb microtitre plate. The plates were incubated at 37°C with medium continuous shaking in a Bioscreen C automated growth curve analyzer (MTX Lab Systems, Vienna, VA, USA). Growth was followed by measuring the OD₆₀₀ at 37°C every 30 min. At pre-determined time points post-

inoculation, the Bioscreen was paused and three 200 μ l aliquots for each condition tested were transferred into a flat bottom 96-well microtiter plate (Microfluor-2 White, Thermo Scientific) and luminescence (in relative light units, RLU) was measured using a Fluoroskan Ascent FL Microplate Fluorometer and Luminometer (Thermo Scientific, Ottawa, Ontario, Canada). Expression levels of each gene of interest in the different strain backgrounds were calculated as RLU/OD₆₀₀ for each time-point.

Construction of a conditional mutant. A fragment (~300-bp) spanning the 5' region of BCAL2641 was PCR amplified, digested by *NdeI* and *XbaI* and cloned into the *NdeI* and *XbaI* digested and dephosphorylated pSC200 plasmid. The plasmids were mobilized into OME12 (Δ BCAM1111-1112) by triparental mating. Transconjugants were selected on LB agar plates containing 100 μ g/ml of trimethoprim (Tp), 200 μ g/ml ampicillin, 10 μ g/ml gentamicin and 0.5% (wt/vol) rhamnose. This strategy creates conditional mutants in which the expression of the targeted gene depended on the rhamnose concentration in the medium (28).

Thin-layer chromatography analyses of polyamines. The conditional mutant and the wild type were grown at 37°C in M9 minimal medium supplemented with final concentrations of Tp 100 μ g/ml and rhamnose 0.4% (wt/vol), permissive condition of expression. An aliquot of an overnight culture in M9 medium with rhamnose was spun down and washed three times with sterile phosphate-buffered saline (PBS), resuspended in PBS, and adjusted to an OD₆₀₀ of 1. Drops (10 μ l) of undiluted suspension and 10-fold serial dilutions were plated onto M9 agar plates supplemented with 0.4% (wt/vol) glucose and incubated at 37°C (non-permissive condition of expression). Bacteria growing on the plates were collected, suspended in sterile PBS, and the OD₆₀₀ was adjusted to 0.1. Polyamines were extracted, derivatized to their dansyl derivatives, sequentially separated on TLC silica gel plates (20×20 cm, Merck, Darmstadt,

Germany) in two solvent systems: I) benzene–triethylamine (20:2 v/v); II) benzene–methanol (10:0.45 v/v) and visualized under ultraviolet light as previously described (12). Standard solutions of putrescine, cadaverine, spermidine and spermine (0.2 mM each) were treated similarly and included as controls.

Catalase enzyme activity assay. Overnight cultures of the wild type *B. cenocepacia* K56-2 in LB were diluted to OD₆₀₀=0.04 into 30 ml fresh LB medium, with or without antibiotics, and incubated at 37°C, 200 rpm for 16 h. Bacterial cells were pelleted, washed with sterile PBS and resuspended in 300 µl (or less if necessary depending on bacterial inhibition of growth by antibiotics) of PBS. The OD₆₀₀ of the bacterial suspensions was measured. The catalase enzyme activity was evaluated using the method described by Iwase et al. (29). Briefly, 100 µl of bacterial suspension or bovine liver catalase solution at different concentrations were added in a glass tube followed by the addition of 100 µl of 1% Triton X-100. Finally, 100 µl of undiluted hydrogen peroxide (30%) were added to the solutions, mixed thoroughly and incubated at room temperature. The height of O₂-forming foam that remained constant for 15 min in the test tube was finally measured using a ruler. The catalase activity of bacterial suspensions was determined using calibration curves constructed using the standard catalase solutions with different concentrations and normalized to the OD₆₀₀ of the tested suspensions.

Statistical Analyses. Unpaired student's t-tests were conducted with GraphPad Prism 5.0.

RESULTS AND DISCUSSION

Putrescine reduces ROS production induced by PmB. Treatment of *B. cenocepacia* K56-2 with 1 mg/ml PmB led to significantly increased production of intracellular ROS, as detected by 2',7'-dichlorofluorescein diacetate (DCF) (Fig. 1). DCF is a colorless, nonfluorescent fluorescein derivative which passively diffuses into cells where the two acetate groups are cleaved by intracellular esterases to yield the non-cell permeable 2', 7'- dichlorofluorescein (30). This cleaved product becomes trapped within the cells and becomes oxidized by intracellular ROS resulting in the formation of a highly fluorescent product; hence it is a measure of generalized oxidant production rather than that of any particular reactive species (30). Lower concentrations of PmB (0.5 mg/ml or less) did not alter the intracellular DCF-detectable ROS pool (data not shown), whereas due to its reduced solubility in the culture medium higher concentrations of PmB could not be reliably tested. Since putrescine protects *B. cenocepacia* from PmB (12), we assessed whether it also alleviates PmB-induced ROS production. Compared to control cells, exogenous putrescine reduced DCF-detectable ROS generation in PmB-treated bacteria (Fig. 1). This effect was assessed at 2 h incubation with PmB and/or putrescine to avoid potential interference from putrescine degradation or metabolic by-products at prolonged incubation times. It should be noted that putrescine did not decrease the background ROS levels produced by bacterial cells not exposed to PmB, but rather caused a slight but significant increase in DCF-detected ROS levels compared to control cells at 20 mM (Fig. 1, white bars). We attributed these results to polyamines catabolism, which also generate ROS (31).

To assess whether endogenous putrescine also has the ability to reduce ROS levels in PmB-treated *B. cenocepacia*, we employed deletion mutants in the putrescine biosynthesis pathway. Putrescine can arise through the action of either ornithine decarboxylase or arginine

234 decarboxylase (12). *B. cenocepacia* has two ornithine decarboxylase homologues, BCAL2641
235 and BCAM1111, and one arginine decarboxylase protein, BCAM1112 (Fig. 2A). The ornithine
236 decarboxylase BCAL2641 is encoded by a gene located on chromosome 1 of *B. cenocepacia*;
237 whereas both the ornithine decarboxylase BCAM1111 and the arginine decarboxylase
238 BCAM1112 are encoded by genes located adjacent to each other, but in opposite orientation, on
239 chromosome 2. In a previous study, we have shown that Δ BCAL2641 had a greater reduction in
240 the amount of secreted putrescine compared to wild type than Δ BCAM1111-BCAM1112 (12).
241 Here, we confirmed that these three enzymes are the only contributors to putrescine production
242 in *B. cenocepacia*. A conditional mutant of BCAL2641 in the Δ BCAM1111-BCAM1112
243 background did not produce detectable levels of putrescine at the non-permissive conditions of
244 expression compared to the wild type strain (Fig. 2B). With respect to the response to PmB, the
245 ornithine decarboxylase BCAL2641 was the only enzyme, among the 3 putrescine synthesis
246 enzymes, involved in resistance against PmB. Δ BCAL2641, but not Δ BCAM1111-BCAM1112,
247 had increased susceptibility to PmB compared to wild type when tested in LB medium (Fig. 2C
248 and 2D) or M9 medium (Fig. 2E). Although the growth of Δ BCAM1111-BCAM1112 was not
249 impaired in LB medium regardless of the initial inoculum size (Fig. 2C and 2D), it exhibited
250 significant reduction in growth compared to the wild type cells in M9 medium (Fig. 2E).
251 Nevertheless, this mutant did not show increased susceptibility to PmB in M9 medium in which
252 its growth was retarded (Fig. 2E). On the contrary, Δ BCAL2641 showed slight reduction in
253 growth in LB medium only at low inoculum size (Fig. 2C) but not at high inoculum size (Fig.
254 2D) or in M9 medium (Fig. 2E). This suggests that these genes involved in putrescine synthesis
255 are not functionally redundant; they seem to be stimulated under different conditions and
256 regulated differently with BCAL2641 only involved in resistance to antibiotics. Next, detection

of ROS by DCF was assessed after incubation of Δ BCAL2641 and Δ BCAM1111-BCAM1112 mutants with PmB for 16 h to allow the different enzymes to reach their maximum expression levels which occurred at about 12 h in the luminescence expression assays (not shown). No differences were observed in PmB-untreated cells between the wild type and the deletion mutants (Fig. 3, white bars). In contrast, Δ BCAL2641 exhibited a significant increase in levels of superoxide and other ROS detected by DCF in response to PmB compared to wild type, whereas Δ BCAM1111-BCAM1112 produced the same level as that in the parental strain (Fig. 3). Together, these results support the notion that putrescine reduces the level of PmB-induced ROS production and this reduction contributes to protection of bacteria from the bactericidal effects of PmB.

Hydroxyl radical is another ROS that may be produced upon oxidative stress. Others have used hydroxyphenyl fluorescein (HPF) to fluorometrically detect hydroxyl radicals upon antibiotic stress (16). Using HPF in similar experiments as above, we found a comparable pattern of reduction of PmB-induced ROS by putrescine (data not shown). However, the fluorescence signal detected by HPF was too low compared to that detected by DCF, and required 100-fold higher inoculum than that for the DCF experiments to detect signal above the background noise of fluorescence. Such high inoculum of cells led to high autofluorescence compared to the actual fluorescence signal detected upon adding HPF, which was not the case with the DCF assays (Fig. S1). Thus, we disregarded the results of HPF assays. Similar criticism to the use of HPF was raised recently concerning the interference between the autofluorescence of cells with the actual fluorescence in the presence of the probe especially upon antibiotic treatment (32).

Although the DCF fluorometric assay is a well established method and has many advantages over other techniques developed for measurement of intracellular ROS (30), the probe may be

nonselective reacting with other oxidants such as hydroxyl radicals and lipid peroxides (33). Hence, to provide additional evidence supporting the DCF fluorometric assays results, we measured the expression of OxyR as an independent indicator of oxidative stress. OxyR belongs to the LysR family of transcription factors whose regulon is involved in the cellular response to oxidative stress (22). OxyR is very sensitive to ROS concentrations, it is activated at very low hydrogen peroxide concentrations leading to upregulation to its regulon (34). Moreover, expression of *oxyR* is upregulated in response to hydrogen peroxide using *lacZ* promoter fusions (35); similarly, another LysR-type transcription regulator involved in the response to oxidative stress is overexpressed in response to ROS (36). Therefore, we constructed derivatives of wild type and mutant strains carrying an *oxyR::lux* promoter fusion to measure *oxyR* gene expression at chromosomal levels. PmB stimulated the *oxyR* expression (Fig. 4A), which was consistent with the induction of intracellular ROS detected by DCF (Fig. 1 and 3). Likewise, catalase activity, regulated by OxyR (22), increased in response to PmB (Table 2). This further confirms the induction of intracellular ROS in response to PmB and validates the findings of DCF fluorometric and *oxyR* expression assays as measures of intracellular ROS. *oxyR* expression was significantly higher in Δ BCAL2641 compared to the parental strain both in the presence or absence of PmB. In contrast, no difference in *oxyR* expression between the wild type and Δ BCAM1111-BCAM1112 was detected in response to PmB (Fig. 4A). No differences in the growth rate of the different strains were noted in absence of PmB; whereas Δ BCAL2641 was more susceptible to PmB compared to the wild type and Δ BCAM1111-BCAM1112 (Fig. S2). These results follow the same pattern of ROS generated in response to PmB in the mutants compared to the wild type strain (Fig. 3).

Next, we investigated the mechanism by which putrescine protects from oxidative stress. Putrescine stimulated the expression of *oxyR* (Fig. 4A), probably as a result of a slight induction of ROS accumulation as detected by DCF (Fig. 1). However, putrescine alleviated the increase in *oxyR* expression in response to PmB (Fig. 4A), suggesting a protective effect against ROS. Nevertheless, putrescine did not induce a statistically significant difference in growth of the wild type in the presence or absence of PmB at this early time point of incubation (3 h) under the conditions of this test (Fig. S2). Supporting the protective role of putrescine from oxidative stress, we confirmed the antioxidant properties of putrescine by demonstrating that it could scavenge superoxide radicals generated *in vitro* from a phenazine methosulfate-NADH system in a concentration dependent manner (Fig. 4B). Together, the results of this section reveal a link between reduced susceptibility to PmB, induction of ROS production, and expression of OxyR with the intracellular level of putrescine, which can be attributed to the antioxidant properties of this polyamine.

Expression of the putrescine synthesis enzymes in response to PmB. To better understand the role of the different putrescine synthesizing enzymes in response to oxidative stress and consequently to PmB, we investigated the expression profiles of their corresponding genes also using *lux* promoter fusions as before. BCAL2641::*lux* gene expression was stimulated by exposure to PmB (Fig. 5A); whereas neither BCAM1111::*lux* nor BCAM1112::*lux* fusions were responsive to PmB (Fig. 5B and 5C respectively). This agrees with the behaviour of Δ BCAL2641 and Δ BCAM1111-BCAM1112 mutants to PmB in terms of antimicrobial resistance (Fig. 2C-2E) and ROS production (Fig. 3). Moreover, this is consistent with our previous data showing increased transcription of BCAL2641, but not BCAM1111 or BCAM1112, in response to PmB (12). BCAL2641 also appears to regulate by an unknown

mechanism the gene expression of BCAM1111 and BCAM1112 putrescine synthesis enzymes, since the expression of both genes was significantly reduced in the Δ BCAL2641 background (Fig. 5B and 5C, respectively). This regulation is not mediated through the action of putrescine since 10 mM of putrescine did not stimulate the gene expression of BCAM1111 or BCAM1112 (not shown). Other indirect regulatory pathways may be involved which will require further investigation. On the other hand, the gene expression of BCAL2641 increased in the absence of BCAM1111 and BCAM1112 (Fig. 5A), which may explain the slight increase in survival of the Δ BCAM1111-BCAM1112 when exposed to PmB shown in Fig. 2E. This might be due to compensation of the reduced synthesis of putrescine by these enzymes being normally stimulated by BCAL2641. Alternatively, BCAM1111 and BCAM1112 might provide feedback inhibition to BCAL2641; thus their absence would lead to increased BCAL2641 gene expression. Notably, the expression of BCAM1112 (RLU/OD₆₀₀ 0.2423) is much lower than that of the other 2 enzymes (RLU/OD₆₀₀ 1.4829 and 1.5585 for BCAL2641 and BCAM1111 respectively). This suggests that *B. cenocepacia* does not preferentially utilize the arginine decarboxylase BCAM1112. This agrees with the fact that *B. cepacia* can degrade arginine only through the use of the succinyl transferase pathway, despite the possession of an arginine decarboxylase homologue (37, 38). Except for Δ BCAL2641, which exhibited reduced growth in the presence of PmB, no differences in growth were observed in the other strains tested regardless of PmB exposure (Fig. S3). Together, our findings expose BCAL2641 as a crucial contributor of putrescine synthesis in the response against antibiotics.

ROS production in response to other bactericidal antibiotics. To evaluate whether the induction of oxidative stress and its amelioration by putrescine is a general phenomenon, we tested other bactericidal antibiotics. Exposure of *B. cenocepacia* to gentamicin, norfloxacin,

ceftazidime and rifampicin led to increased ROS production as detected by DCF (Fig. S4) at sub-lethal concentrations; i.e. concentrations below but more specifically near the MIC of these antibiotics (Fig. S5). Putrescine reduced the antibiotic-induced elevation in ROS levels only for norfloxacin and rifampicin (Fig. 6), and this correlated with induction of BCAL2641 gene expression (Fig. 7A and 7B, respectively). This agrees with the contribution of BCAL2641 in resistance to both antibiotics that we have previously reported (12). Moreover, *oxyR* transcription was also upregulated in response to both norfloxacin and rifampicin (Fig. 7A and 7B, respectively), which was reflected in an increase in the catalase activity (Table 2), supporting the notion that both antibiotics lead to increased ROS production (Fig. 6 and Fig. S4). In contrast, neither antibiotic affected BCAM1111 gene expression (Fig. 7), indicating that this gene and its product are not directly involved in the response to antibiotic-mediated oxidative stress. It should be noted that higher rifampicin concentrations resulted in great reduction in the expression of BCAL2641, *oxyR*, and BCAM1111 (Fig. 7B), which might be attributed to non-specific inhibition of transcription by rifampicin, especially at 512 µg/ml where expression from these genes was almost completely inhibited.

Putrescine did not reduce ROS production generated in response to ceftazidime, but rather further increased the generated ROS at 10 mM but not at 20 mM concentration of putrescine (Fig. 6). Ceftazidime did not affect the expression of BCAL2641, *oxyR* or BCAM1111 (Fig. 7C), and did not alter the catalase enzyme activity (Table 2). However, in a previous study we reported that BCAL2641 is involved in the response of *B. cenocepacia* to ceftazidime (12). This may suggest another role of BCAL2641 in the protective actions against ceftazidime not related to the oxidative stress.

Concerning the response to gentamicin, exogenous putrescine did not affect the level of gentamicin-induced superoxide anion (Fig. 6). Moreover, gentamicin did not alter the expression of BCAL2641 (Fig. 7D). This agrees with the previously reported lack of involvement of this enzyme in the response to gentamicin in *B. cenocepacia* (12). Furthermore, gentamicin did not affect the expression of *oxyR* (Fig. 7D). However, the highest tested concentrations of gentamicin did reduce the expression of both BCAL2641 and *oxyR* (Fig. 7D). Similarly, gentamicin reduced the catalase enzyme activity (Table 2). Such inhibition might be due to the mechanism of action of the aminoglycoside inhibiting translation and protein synthesis in general, since it also inhibited the expression of BCAM1111, which consequently might have led to increased ROS levels at high concentration (Fig. 6).

Conclusions. We show that: (i) sub-lethal concentrations of different bactericidal antibiotics (PmB at 1 mg/ml, rifampicin at 256 and 512 µg/ml and norfloxacin at 32 and 64 µg/ml) induce oxidative stress in *B. cenocepacia* that is manifested as induction of ROS formation as detected by DCF, stimulation of expression of the transcription regulator OxyR involved in response to oxidative stress (at antibiotic concentrations similar to or even lower than those inducing ROS formation; PmB at 0.5 mg/ml, rifampicin at 16 and 32 µg/ml and norfloxacin at 16-64 µg/ml), and increased catalase enzyme activity (PmB at 0.5 mg/ml, rifampicin at 16 µg/ml and norfloxacin at 8 µg/ml); (ii) this response does not apply to gentamicin and ceftazidime which do not induce OxyR expression or increase catalase enzyme activity, suggesting that not all bactericidal antibiotics induce oxidative stress; (iii) putrescine protects against oxidative stress induced by several bactericidal antibiotics (PmB, norfloxacin and rifampicin); (iv) protection by putrescine correlates with increased BCAL2641 gene expression; (v) BCAL2641, in addition to synthesizing putrescine, regulates the other putrescine biosynthetic enzymes BCAM1111 and

BCAM1112 by an unknown mechanism that does not directly involve putrescine. Together, these observations suggest a model (Fig. 8) by which *B. cenocepacia* responds to antibiotic stress by overproducing putrescine and in turn, this polyamine protects bacterial cells by a surface effect blocking antibiotic binding (12) as well as by reducing oxidative damage.

Putrescine was previously shown to communicate antibiotic resistance among different bacteria (12). Its increased production in *B. cenocepacia* occurs in response to a subset of bactericidal antibiotics (12), which induce oxidative stress in bacterial cells at near lethal concentration ranges. It is still controversial whether the generation of ROS is the cause of lethality of antimicrobial agents or a consequence of antibiotic stress (16, 20, 21). However, it is conceivable that the oxidative stress accompanying antibiotic treatment imposes a metabolic burden on the bacterial cells at near death conditions. Thus, our results demonstrating a protective role for putrescine in the response to the oxidative stress generated in *B. cenocepacia* during antibiotic exposure represent another mechanism of protection from the antibacterial effects of bactericidal antibiotics. This agrees with previous reports on the antioxidant properties and protective effects of putrescine against antibiotic induced ROS formation in *E. coli* (17).

While little is known about the physiological levels of putrescine, it seems that its level varies in different body sites. For example, putrescine concentration was reported to be 3 mM in urine (11), whereas it was shown to be up to 0.2 mM in sputum samples from CF patients (39, 40). However, it is difficult to predict the local concentration of putrescine and other polyamines in the lung of CF patients, as infection alters the rheology of the mucus and the lung environment (41). Moreover, putrescine levels increase dramatically (by 10 fold or more) during exacerbations of bacterial infections in CF patients (39, 40). Hence, the concentrations used in this study could potentially resemble the physiological situation in certain body compartments.

Furthermore, a direct relationship exists between increased putrescine concentration during infection and the proliferation of lung microbiota and specific pathogens such as *P. aeruginosa* in the lungs of CF patients (40). Also, putrescine and other polyamines in genital mucosal fluids increase the resistance of *N. gonorrhoeae* to antimicrobial peptides (PmB and LL-37), possibly enhancing its survival during infection by reducing bacterial susceptibility to host-derived antimicrobials (11). Interestingly, the expression of the ornithine decarboxylase BCAL2641 is induced in *B. cenocepacia* in CF conditions compared to soil environmental like conditions shown by comparative transcriptomics, underscoring the importance of putrescine, and this enzyme in particular, during infection (42).

This study also provides new information on the regulation of the putrescine synthesis enzymes. The ornithine decarboxylase BCAL2641 gene responds to the external antibiotic signals, while the other ornithine decarboxylase BCAM1111 or the arginine decarboxylase BCAM1112 do not. Also, BCAL2641 regulates the expression of BCAM1111 and BCAM1112 since their expression depends on the presence of BCAL2641. This suggests that upon antibiotic stress maximal production of putrescine is required, which arises from the upregulation of BCAL2641 and by maintaining the expression of the other two enzymes in a BCAL2641-dependent manner. The molecular mechanism of this regulation awaits further investigation.

In conclusion, this study broadens our understanding on the mechanism of chemical communication of antibiotic resistance mediated by putrescine. In addition, it provides a clear target for the design of inhibitors targeting the ornithine decarboxylase BCAL2641 that is critically implicated in this phenomenon. Such inhibitors would not only reduce the resistance to antibiotics in *B. cenocepacia* but also would reduce its ability to communicate high-level resistance to other less resistant bacteria.

439

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445

TABLE 1 Strains and Plasmids

Strain or plasmid	Relevant characteristics ^a	Source and/or reference
Strains		
<i>Burkholderia cenocepacia</i>		
K56-2	ET12 clone related to J2315, CF clinical Isolate, ^b BCRRC	(43)
OME11	K56-2, ΔBCAL2641	(12)
OME12	K56-2, ΔBCAM1111-ΔBCAM1112	(12)
OME49	OME12 <i>P_{rha}</i> ::BCAL2641	This study
OME50	K56-2, <i>P_{BCAL2641}</i> ::pGSVTp- <i>luxCDABE</i> ; Tp ^R	This study
OME51	OME12, <i>P_{BCAL2641}</i> ::pGSVTp- <i>luxCDABE</i> ; Tp ^R	This study
OME52	K56-2, <i>P_{BCAM1111}</i> ::pGSVTp- <i>luxCDABE</i> ; Tp ^R	This study
OME53	OME11, <i>P_{BCAM1111}</i> ::pGSVTp- <i>luxCDABE</i> ; Tp ^R	This study
OME54	K56-2, <i>P_{BCAM1112}</i> ::pGSVTp- <i>luxCDABE</i> ; Tp ^R	This study
OME55	OME11, <i>P_{BCAM1112}</i> ::pGSVTp- <i>luxCDABE</i> ; Tp ^R	This study
OME56	K56-2, <i>P_{oxyR}</i> ::pGSVTp- <i>luxCDABE</i> ; Tp ^R	This study
OME57	OME11, <i>P_{oxyR}</i> ::pGSVTp- <i>luxCDABE</i> ; Tp ^R	This study
OME58	OME12, <i>P_{oxyR}</i> ::pGSVTp- <i>luxCDABE</i> ; Tp ^R	This study
<i>Escherichia coli</i>		
DH5α	F ⁻ φ80 <i>lacZ</i> M15 <i>endA1 recA1 supE44 hsdR17</i> (r _K ⁻ m _K ⁺) <i>deoR thi-1 nupG supE44 gyrA96relA1</i> Δ(<i>lacZYA-argF</i>)U169, λ ⁻	Laboratory stock
GT115	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80Δ <i>lacZ</i> ΔM15 Δ <i>lacX74 recA1 rpsL</i> (StrA) <i>endA1</i> Δ <i>dcm uidA</i> (ΔMluI):: <i>pir-116</i> Δ <i>sbcC-sbcD</i>	Invivogen
Plasmids		
pRK2013	<i>ori_{colE1}</i> , RK2 derivative, Kan ^R , <i>mob</i> ⁺ , <i>tra</i> ⁺	(26)
pGSVTp- <i>lux</i>	Mobilizable suicide vector containing the <i>lux</i> operon, derivative from pGSV3- <i>lux</i> (44); OriT; Tp ^R	(45)
pSC200	<i>oriR6K</i> , <i>P_{RhaB}</i> rhamnose-inducible promoter, Tp ^R , <i>mob</i> ⁺	(28)
pOE14	<i>P_{BCAL2641}</i> :: <i>luxCDABE</i> transcriptional fusion in pGSVTp- <i>lux</i>	This study
pOE17	<i>P_{rha}</i> ::BCAL2641 in pSC200	This study
pOE18	<i>P_{BCAM1111}</i> :: <i>luxCDABE</i> transcriptional fusion in pGSVTp- <i>lux</i>	This study
pOE19,	<i>P_{BCAM1112}</i> :: <i>luxCDABE</i> transcriptional fusion in pGSVTp- <i>lux</i>	This study
pOE20	<i>P_{oxyR}</i> :: <i>luxCDABE</i> transcriptional fusion in pGSVTp- <i>lux</i>	This study

^aTp^R, trimethoprim resistance, Kan^R, kanamycin resistance, Tet^R, tetracycline resistance.

^bBCRRC, *B. cepacia* Research and Referral Repository for Canadian CF Clinics.

TABLE 2 Catalase enzyme activities.

Antibiotic Concentration (µg/ml)	Catalase Activity* % Units/OD ₆₀₀ (SEM)	Difference from control (P-value)
None	100 (1.3)	Not applicable
Polymyxin B (500)	120.1 (4.2)	0.0002
Norfloxacin (8)	110.6 (1.2)	0.0012
Rifampicin (16)	134.5 (5.2)	<0.0001
Ceftazidime (32)	99.8 (12.4)	0.978 (Not significant)
Gentamicin (1000)	48.0 (7.1)	<0.0001

* Results from 2 independent experiments, n=6. r^2 of calibration curves was: 0.9644 and 0.9544.

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Figure Legends

FIG 1. Putrescine reduces ROS production induced by PmB in *B. cenocepacia* K56-2. ROS were detected by DCF. n= 6 from 2 independent experiments.

FIG 2. BCAL2641 is the only putrescine synthesis enzyme in *B. cenocepacia* involved in reduced susceptibility to PmB. A. Putrescine synthesis pathway in *B. cenocepacia* K56-2 together with the enzymes involved. ADC, arginine decarboxylase; ODC, ornithine decarboxylase. B. TLC plate showing the lack of production of putrescine in Δ BCAM1111-1112 *P_{rha}*-BCAL2641 conditional mutant under non-permissive conditions. Put, putrescine; Cad, cadaverine; Spd, spermidine; Spn, spermine. C-E. Sensitivity of wild type and putrescine synthesis mutants Δ BCAL2641 (OME11) and Δ BCAM1111-1112 (OME12) to 2048 μ g/ml PmB determined turbidimetrically. n=3 from a representative experiment. C, low initial inoculum in LB medium; D, high initial inoculum in LB medium; D, in M9 minimal medium.

FIG 3. BCAL2641 is the main ornithine decarboxylase responsible for reduction of ROS accumulation and reduced susceptibility to PmB. ROS production in response to 1 mg/ml PmB in wild type K56-2, compared to putrescine synthesis mutants Δ BCAL2641 (OME11) and Δ BCAM1111-1112 (OME12) detected by DCF. n=6 from 2 independent experiments.

FIG 4. A. Induction of OxyR expression as an indicator of ROS accumulation in the wild type (OME56) compared to putrescine synthesis mutants Δ BCAL2641 (OME57) and Δ BCAM1111-1112 (OME58) in response to 500 μ g/ml PmB with or without 10 mM Put determined by luciferase expression assay at 3 h. Results are shown as percentage of relative light units RLU/OD₆₀₀ relative to the OME56 control (K56-2 background). The mean RLU/OD₆₀₀ of the control is 0.09567. The percentages of OD₆₀₀ are shown in Fig. S2. n=9 from 3 different clones.

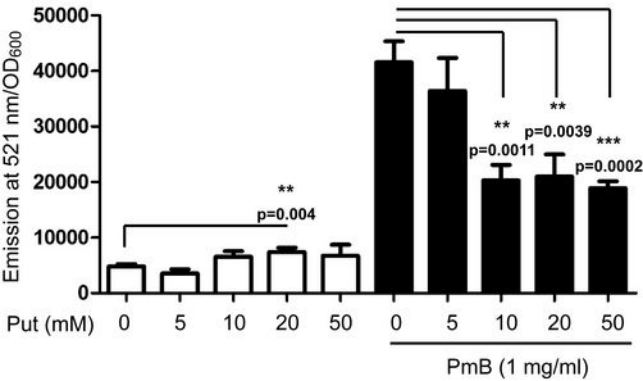
* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. B. *In vitro* antioxidant activity of putrescine. $n=6$ from 2 independent experiments.

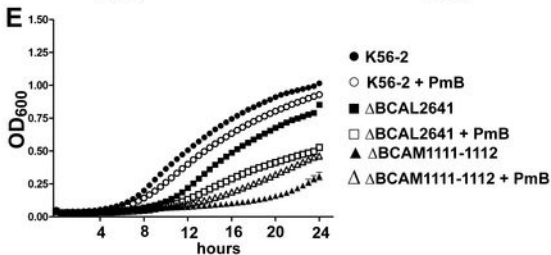
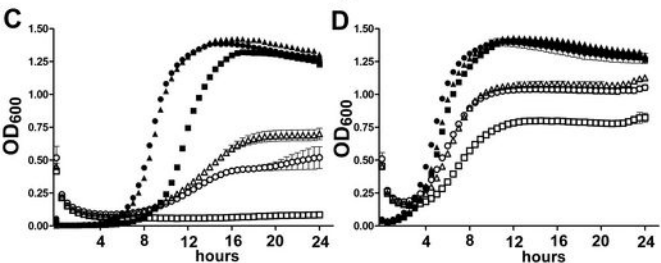
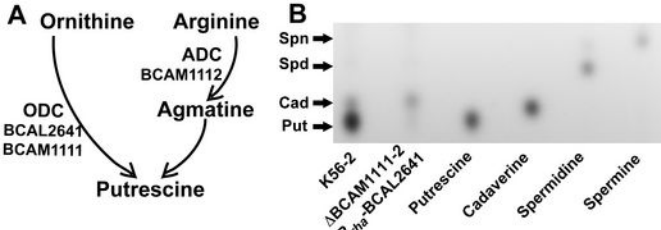
FIG 5. Luciferase expression assay of the different putrescine synthesizing enzymes in response to 500 $\mu\text{g/ml}$ PmB at 3 h. Results are shown as percentage of relative light units RLU/OD₆₀₀ relative to the control (untreated K56-2 background). The percentages of OD₆₀₀ are shown in Fig. S3. A Expression of BCAL2641 in the wild type (OME50) and $\Delta\text{BCAM1111-1112}$ (OME51) backgrounds. $n=6$ from 2 different clones. The mean RLU/OD₆₀₀ of the control is 1.4829. B Expression of BCAM1111 in the wild type (OME52) and $\Delta\text{BCAL2641}$ (OME53) backgrounds. $n=6$ from 2 different clones. The mean RLU/OD₆₀₀ of the control is 1.5585. C Expression of BCAM1112 in the wild type (OME54) and $\Delta\text{BCAL2641}$ (OME55) backgrounds. $n=7$ from 2 different clones. The mean RLU/OD₆₀₀ of the control is 0.2423. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

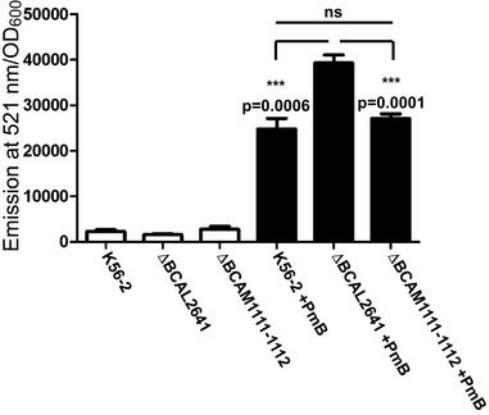
FIG 6. The role of putrescine in the bactericidal antibiotics-mediated ROS accumulation in *B. cenocepacia* K56-2. $n=9$ from 3 independent experiments. The 4 tested antibiotics alone significantly ($p < 0.001$) induced the accumulation of ROS compared to control cells. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

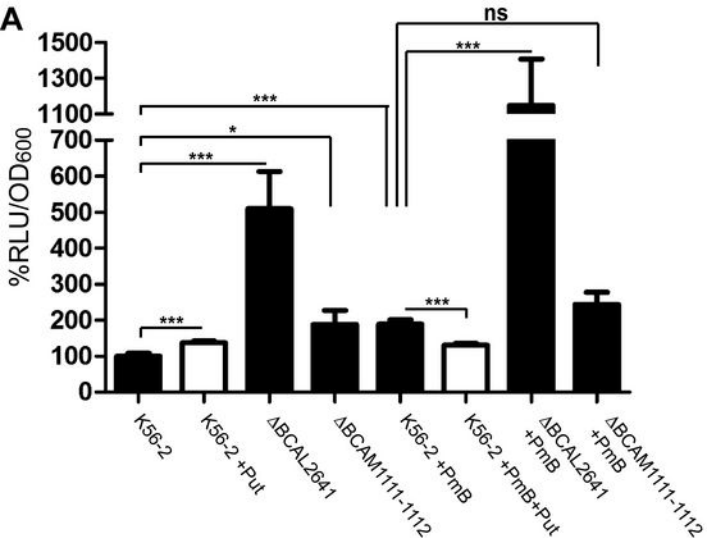
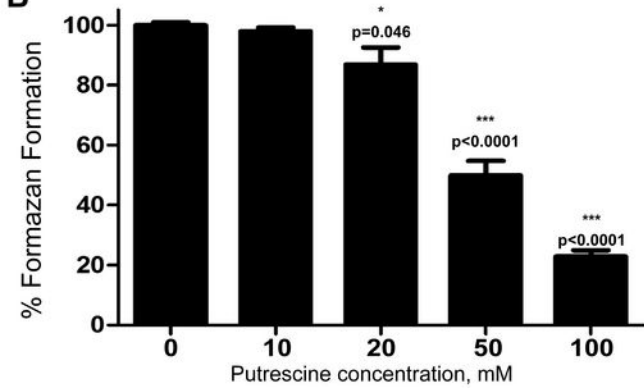
FIG 7. Effect of different antibiotics on the expression of BCAL2641 (in OME50), *oxyR* (in OME56), and BCAM1111 (in OME52) determined using a luciferase expression assay at 3 h. Results are shown as percentage of relative light units RLU/OD₆₀₀ relative to the control (untreated K56-2 background). The percentages of OD₆₀₀ are shown in Fig. S5. n = a minimum of 6 from at least 2 different clones. The mean RLU/OD₆₀₀ of the control is 1.0759 for BCAL2641; 0.1087 for *oxyR* and 1.4723 for BCAM1111. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

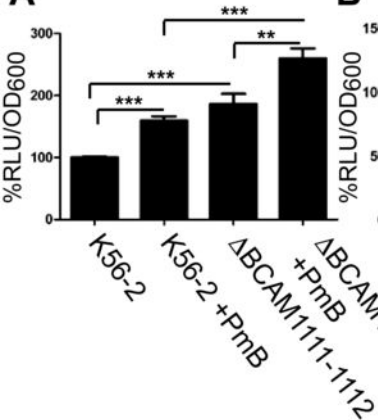
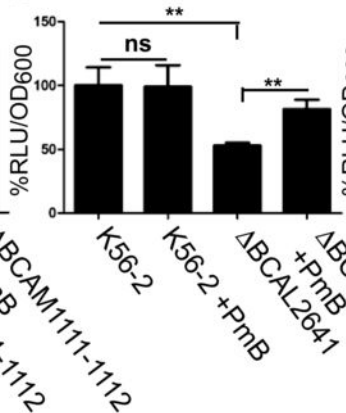
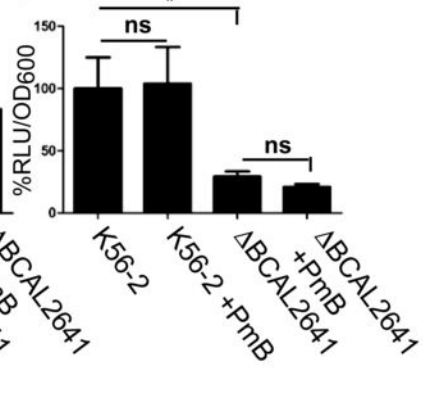
FIG 8. Model summarizing the role of putrescine in protecting *B. cenocepacia* from antibiotic-induced stress.

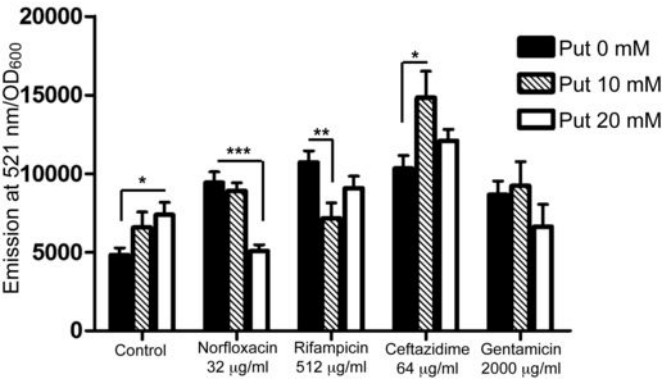


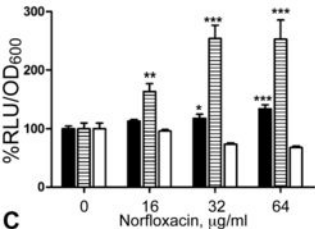
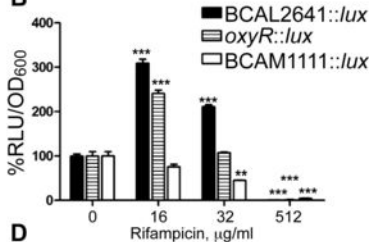
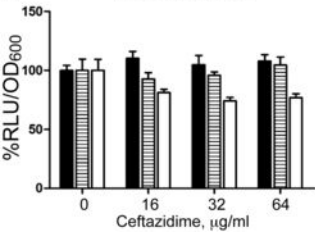




A**B**

A*BCAL2641::lux***B***BCAM1111::lux***C***BCAL1112::lux*



A**B****C****D**